A “BOTTOM-UP” APPROACH TO NEUROSCIENCE; the Role of the Gut-Brain Axis in Mental Health and Cognitive Processes

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ACKNOWLEDGEMENTS

I'd like to thank everyone from Bowdoin College who made this experience possible by supporting me during the Fulbright application process: Janice Jaffe, my application mentor, Patsy Dickinson and Jack Bateman, my research advisors, and Jens Klenner and Jill Smith, my German professors. As always, I am also thankful for my parents who always encouraged curiosity and wonder at the natural world. They are the reason I have pursued science. I would also like to thank my research advisor in Graz, Aitak, who was a wonderful mentor and a scientist that I greatly admire.

Finally, to anyone suffering from mental illness:
We are making progress. So much progress. Researchers are taking the complexity and individuality of mental health very seriously, and we will find solutions.

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ABSTRACT

The “gut-brain axis” describes the bidirectional communication between the gastrointestinal tract (GI) and the central nervous system. Gut microbiota are one of the primary drivers of gut-brain communication. The gut microbiome can influence the biochemistry and functionality of the brain and vice versa, leading us to believe that many mental health conditions are affected by the gut-brain axis and intestinal microbiota compositions. For my Fulbright research project, I took a “bottom-up” approach to understanding the gut-brain communication. One topic I researched was how the gut-brain axis is involved in the presentation of bipolar disorder in mice. I found that fecal microbiota transplants (FMT) from a human patient with bipolar disorder causes mice to exhibit more anxiety-related-bahavior than mice with FMT from a healthy control. This research has the potential to help us understand the role of the gut-brain axis in mental health conditions like bipolar disorder, and could play an important role in the development of new treatments. I also focused on the ways in which the gut-microbiota regulated peptide, peptide YY (PYY), affects cognitive processes. To do this, I looked into how a lack of PYY signaling in mice influences spatial learning and memory, as well as emotional response to immune stress. I found that Pyy-Knockout (KO) mice lacking the Pyy gene tended to have worse spatial learning and memory abilities than WT mice. Although more experiments need to be done, I also found that immune stress from dextran sulphate sodium (DSS) induced colitis may cause Pyy-KO mice to express more anxiety-behavior than WT mice.

INTRODUCTION

The Gut-Brain Axis

Ever had a gut feeling? In recent years, it has been revealed that the gastrointestinal (GI) tract and the central nervous system (CNS) are able to communicate bidirectionally through what is known as the “gut-brain axis” (GBA). As a result, the workings of the gut can influence the biochemistry and functionality of the brain and vice versa. Until the discovery of the gut-brain axis, the importance of the gut had been largely overlooked, but now scientists have begun to take a “bottom up” approach to solving the mysteries of the brain.

Many communication pathways exist between the gut and the brain. One primary mechanism through which the GI tract and the brain interact is via the vagus nerve, which extends from the dorsal motor nucleus in the medulla through the neck, chest, abdomen and colon. The vagus nerve is responsible for parasympathetic control of heart rate, respiratory rate, swallowing, digestion, immune responses, and transmitting signals between the digestive tract and the brain.[1] The gut-brain axis also includes the central nervous system, neuroendocrine system, neuroimmune systems, the hypothalamic-pituitary-adrenal axis (HPA axis), the autonomic nervous system, and the enteric nervous system (Fig.1).

Interactions between the vagus nerve and the enteric nervous system (ENS) play a large role in gut-brain communication. The ENS is the gut’s nervous system; it consists of a neural plexus that branches across the GI tract from the esophagus to the anus.[1] The ENS is composed of roughly 100 million neurons, and is similar to the brain in both structure and function, giving it the nickname, “the second brain.” [2] In the intestines, the ENS regulates nutrient detection, intestinal motility, sensory input reactions, and immune responses. The ENS interacts with the vagus nerve by releasing neurotransmitters, hormones, and peptides that can cross the blood brain barrier (BBB) and be intercepted by the vagus nerve.[1]

The Microbiome

The human body is host to trillions of microbes. This community of microorganisms is known as the "microbiome." Within the first moments of life, we begin to supply our microbiomes with bacteria from our mother's birth canal, the environment, and our diet. Other mammals acquire their microbiomes in the same way. Bacteria are everywhere in and on our bodies: our skin, our mouths, and our intestines. Although some bacteria are pathogenic, most of the bacteria that make up our microbiomes are commensal, and many are even essential to our health and functionality.

The gut microbiome is a major focus in the study of the mammalian microbiome. The human intestine alone contains 100 trillion bacteria. These microbes play an important role in the workings of the gut-brain axis, and are essential to our health and how we function and behave.[3] Commensal, pathogenic, and probiotic bacteria in the gastrointestinal (GI) tract all influence the brain by way of neuroimmune, neuroendocrine, and neural pathways. Populations of intestinal microbes produce signaling molecules such as hormones, neurotransmitters, and monoamines that affect central nervous system (CNS) activity via the vagus nerve.[1] As a result, changes in intestinal microbial composition known as "gut dysbiosis" may alter the levels of these signaling molecules in the brain, affecting neural function and behavior. This makes gut microbiota a point of interest when it comes to the study of mood disorders and cognition. Many neurological disorders such as anxiety, depression, bipolar disorder, autism, and schizophrenia are all linked to gut dysbiosis.

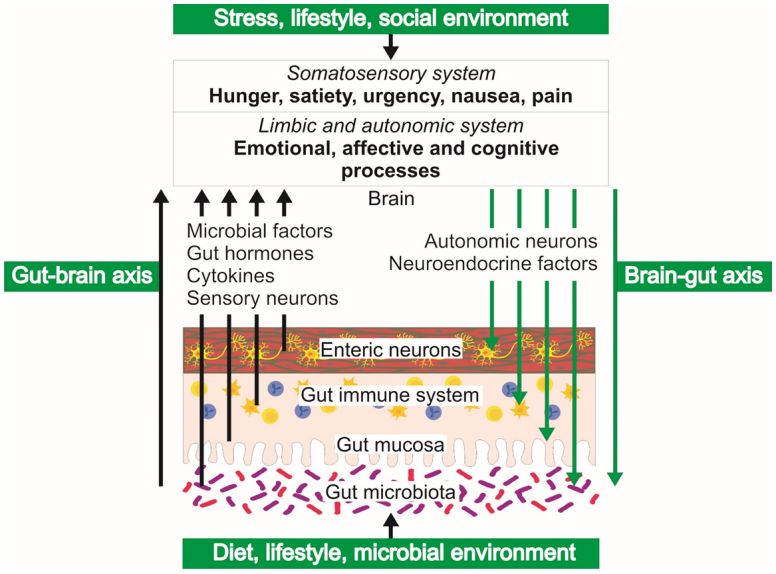

Figure 1. The "gut-brain axis" describes the bidirectional communication between the gut and the brain. The gut-brain axis includes the central nervous system, neuroendocrine system, neuroimmune systems, the hypothalamic-pituitary- adrenal axis (HPA axis), the autonomic nervous system, and the enteric nervous system. (Image created by © Judith C.J. Holzer).

MY RESEARCH PART I: Mood Disorders and the Gut-Brain Axis

Depression and the Gut-Brain Axis

Depression, also known as major depressive disorder or clinical depression, is a debilitating mental health disorder that affects people's thoughts, mood, feelings, behavior, and motivation. Those who suffer from depression typically experience low moods, a poor sense of well-being, and an aversion to activity. In extreme cases, depression can lead to suicide ideation and attempt. According to the World Health Organization (WNO), over 280 million people suffer from depression worldwide.

There are many potential causes of depression. Depression is correlated with changes in the brain such neurotransmitter imbalance, impaired neurogenesis, neuroinflammation, reduced neuroplasticity, and disrupted neuronal circuitry.[4,5] Depressive episodes are largely due to disturbances in the hypothalamic-pituitary adrenal (HPA) axis system, which is a network of endocrine pathways controlled by negative feedback loops between the hypothalamus, pituitary gland, and adrenal gland.[6] There is likely a large genetic component in the onset of major depressive disorder, with 37% to 48% heritability.[7,8] Environmental factors are also at play in triggering the brain changes that cause depression.

Typical treatments for depression such as drugs and psychotherapy all target the brain. However, like many other mood disorders, depression is also influenced by the gut-brain axis. Gut microbiota have been found to affect the regulation and development of the HPA axis,[9] the function of the immune system,[10] the construction of the blood–brain barrier,[11] neurotransmitter synthesis and recognition,[12] neurogenesis,[13] neuralgia development,[14,15] and the myelination of neurons.[16] Studies have found that patients with depression have different gut microbiota compositions from healthy controls, with depressed individuals typically exhibiting less diversity and richness in gut bacterial taxa.[17,18] Abnormal gut microbiota could be the result of numerous factors including antibiotic treatments, diet, stress[19–23], and genetics. Such findings indicate that the regulation of gut microbiota could be a potential treatment for depression.

One way that gut microbiota regulation could be used to treat depression is through fecal microbiota transplantation (FMT). FMT has been used successfully as a

treatment for *Clostridium difficile* infection, inflammatory bowel disease (IBD), and ulcerative colitis.[24] Researchers have found that symptoms of depression are transferable via FMT in mice. Mice receiving fecal transplants from human patients suffering from depression displayed more depressive symptoms than mice receiving transplants from a healthy control.[25] Other studies have shown that FMT from healthy human donors can alleviate symptoms of depression and anxiety in mice.[26] FMT from a healthy donor into the gut of a person with depression or another gut-brain axis related mental disorder could be a promising treatment. In a recent study, human patients suffering from depression were treated with FMT from healthy donors in addition to taking their usual medication and attending psychotherapy. Both patients in the study reported less depressive symptoms 4 weeks after FMT, and in one patient, these effects lasted up to 8 weeks.[27] Although preliminary, these results demonstrate the potential of FMT as (at least) an adjunctive treatment for depression and other mental disorders.

Bipolar Disorder and the Gut-Brain Axis

Bipolar disorder (BD) is a condition that causes atypical changes in mood, energy-level, and activity. People suffering from BD typically experience mood swings with depressive lows and manic highs. As a result, BD is categorized by both depressive and manic episodes. Like depression, BD is influenced by the gut-brain axis. Studies have found that BD patients harbor different bacteria populations and have lower microbial diversity than healthy controls.[28] However, the connection between BD and the gut-brain axis has not been extensively researched. As with depression, it is possible that FMT could offer solutions for people suffering from BD.

The Effect of Human Bipolar Fecal Microbiota Transplantation on Mouse Behavior

Currently, there is no good mouse model for bipolar disorder. Additionally, gut microbiota based treatments for BD have not been studied as extensively as they have for depression. As part of my Fulbright research project, I looked into how the gut-brain axis is involved in the development and behavioral manifestation of bipolar disorder in mice. To do this, I studied how fecal microbiota transplants (FMT) from a human donor in a mixed episode with a YMRS score of 14 and a HAM-D score of 20 affected mouse

behavior as compared to mice with transplants from a healthy weight and age matched control with HAM-D and YMRS scores of 0. I used an elevated plus maze (EPM) and light/dark box test (LDT) to test for anxiety-related-behavior, the splash test and forced swim test (FST) to evaluate depression-like-behavior, and the social interaction test (SIT) to assess differences in social behavior between bipolar and control groups. This work has the potential to provide a better mouse model for BD as well as to shed light on how FMT could be involved in BD treatments.

MY RESEARCH PART II: Peptide YY and the Gut-Brain Axis

The NPY Family

The neuropeptide Y (NPY) family of biologically active peptides (Fig) includes neuropeptide Y (NPY), peptide YY (PYY), and pancreatic polypeptide (PP). Members of the NPY family are released throughout the gut-brain axis, making them of particular interest when it comes to the study of bidirectional gut-brain communication. PYY and PP are produced by endocrine L cells in the gut and in pancreatic islets,[29] and NPY is released at all levels of the gut-brain axis, including systems such as enteric neurons, primary afferent neurons, sympathetic neurons, and several other neuronal pathways in the brain. Both NPY and PYY influence gut-brain signaling by inhibiting gastrointestinal motility and electrolyte secretion.[30] PYY affects signaling in the brain by binding to a family of receptors called Y receptors (Fig. 2).[31]

PYY and Gut Microbiota

There is a strong relationship between gut microbiota and peptide YY (PYY). Gut microbiota influence PYY levels by regulating both its secretion from enteroendocrine cells and gene expression.[32,33] Bacteria directly affect PYY secretion by releasing metabolites and biomolecules that can bind to functional toll-like receptors expressed by enteroendocrine cells.[33,34] Intestinal microbiota also produce short chain fatty acids (SCFA) by fermentation of dietary fibers. In humans, these SCFAs, particularly propionate and butyrate, have been found to regulate PYY gene expression.[35] In mice,

a lack of PYY gene expression has been shown to lead to diet-dependent changes in gut-microbiome composition.[36]

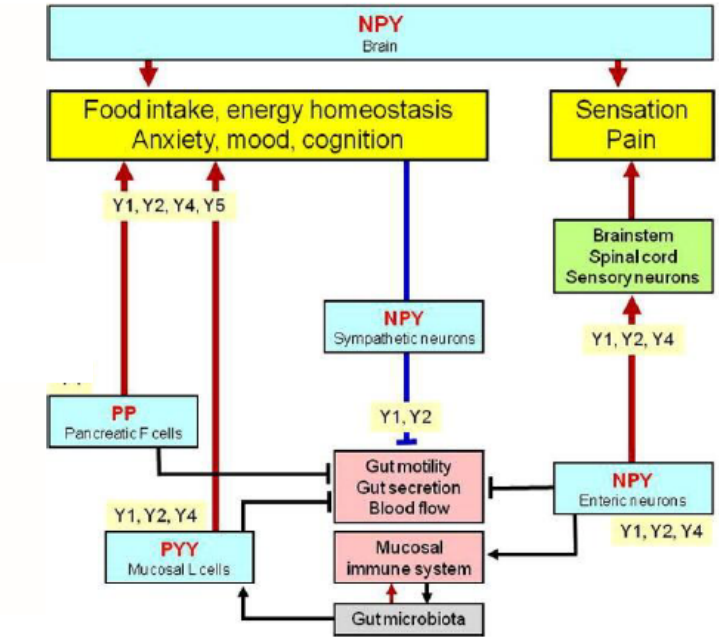

Figure 2. Peptide YY is regulated by gut-microbiota and can affect multiple systems of the brain such as food intake, energy homeostasis, anxiety, mood, and cognition by binding to Y receptors.

The Role of PYY in Cognitive Processes

As part of my research, I set out to assess the ways in which peptide YY (PYY) affects brain functionality and cognitive processes. I evaluated the spatial learning and memorization abilities of Pyy-Knockout mice lacking the Pyy gene (Pyy-KO) with a Barnes Maze (BM) and novel object recognition test (NORT).

PYY and Emotional Response to Immune Stress

There is a strong relationship between mental health and the immune system. Internal immune stress can cause the pathogenesis of various mood disorders.[37] Previous research in my lab demonstrated that PYY may play a role in the ability of gastrointestinal inflammation to influence emotional behavior in mice.[38] I sought to contribute to these findings by comparing anxiety-behavior in Pyy-KO and WT mice with dextran sulfate sodium (DSS) induced colitis. In humans, colitis can be caused by inflammatory bowel disease, which is a condition that causes chronic inflammation of the GI tract. Crohn's disease and ulcerative colitis are the two types of IBD. Because of

the gut-brain axis, IBD implicates the brain as well as the gut, and psychological stress has been found to trigger IBD or cause relapses. Additionally, people with psychological disorders such as depression and anxiety are more likely to develop IBD.[4] As a result, we believe that a lack of PYY signaling in mice would affect their emotional behavior during immune stress.

MATERIALS & METHODS

Animal Models and Maintenance

This procedure was adapted from Farzi et al. 2021.[39] All experiments were approved by an ethical committee at the Federal Ministry of Education, Science and Research of the Republic of Austria. Male mice were used for all Pyy-knockout (KO) experiments. Pyy-KO mice were generated by removing the entire Pyy coding sequence including the initiation start as previously described. Mice were originally generated on a C57Bl/6-129/SvJ strain background, but backcrossed onto the C57BL/6 strain for at least four generations. Female C57BL/6 mice were used for bipolar fecal microbiota transplant (FMT) experiments. Mice were housed at a controlled temperature (22°C) and illumination (12:12 hr light-dark cycle, lights on at 07:00 am), and given unlimited access to water and a standard chow diet.

Fecal Microbiota Transplants (FMT)

To prepare for fecal microbiota transplantation (FMT) from the human donors, female C57BL/6 mice were treated with an antibiotics cocktail (100 mg neomycin, 200 mg meropenem, and 60 mg vancomycin mixed in 200 mL of tap water and stored at 4°C) for 1 week in order to deplete their existing gut microbiome and promote the uptake of donor microbiota. During the antibiotics course, mice were weighed daily to monitor their health. If a mouse lost more than 20% of its original body weight, it was removed from the experiment and re-caged with chow and regular water. One day before FMT, mice were not allowed to feed (fasting phase) in order to prevent fecal blockage in the colon during the transplantation procedure. During this fasting period, mice were given normal tap water instead of the antibiotics solution but no food. On the day of the FMT

procedure, fecal pellets were collected from each mouse, placed on dry ice in a labeled tube, and then stored at -80°C for further analysis.

The Young Mania Rating Scale (YMRS) was used to assess the severity of manic symptoms (Young et al., 1978), and the Hamilton Depression Scale for Depression (HAM-D) was used to assess the severity of depressive symptoms (Hamilton, 1960) in human donors. Fecal samples were collected from a female human donor in a mixed episode of bipolar disorder with a YMRS score of 14 and a HAM-D score of 20, as well as from a weight and age matched healthy control with HAM-D and YMRS scores of 0. The bipolar donor was on a low dose of venlafaxine. A donor not on medication was unable to be found during the recruitment period. Within 6 hours of collection, fecal samples were processed with PBS, 10% glycerin (to prevent freezing damage), and stored in glass anaerobic tubes (Hungate Culture Tubes, Chemglass Life Sciences) at -80°C. Samples were taken out of the freezer and thawed two hours before the FMT procedure.

The FMT procedure itself took place in the hood. While anesthetized with isoflurane, one group of mice received a stool sample from the bipolar donor and another group of mice was transferred a stool sample from the healthy control. Approximately 0.2 mL of human stool sample was administered rectally with a plastic gavage needle into each mouse. A few drops of stool were also placed in the cage and on a few chow pellets in order to promote further microbiota uptake. The health of the mice were assessed 24 hours post FMT.

In order to test for anxiety-like-behavior, both control and bipolar mice were subjected to a series of behavioral tests. The experiment was performed on three different cohorts of mice. In cohort 1, the LabMaster system was employed to assess sucrose preference, locomotion, exploration, and food and water intake for a one week period starting 7 days post FMT. During cohort 2, the social interaction test (SIT) was performed 10 days post FMT and the forced swim test (FST) and a Splash Test (ST) were performed 11 days post FMT. Six separate mice from cohort 2 were also subjected to the LabMaster system as in cohort 1. In cohort 3, the light/dark box test (LDT) was performed 11 days post FMT and the elevated plus maze (EPM) was performed 13

days post FMT. As in cohort 2, six separate mice were also put in the LabMaster cages(Fig. 3).

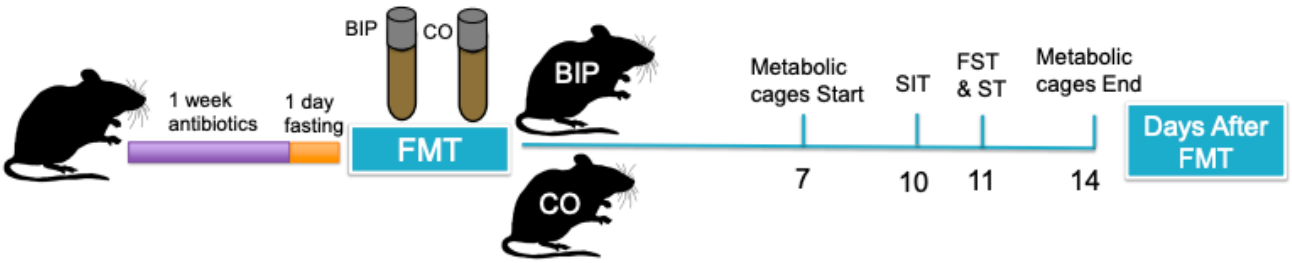

Figure 3. Visual representation of the cohort 2 protocol for fecal microbiota transplantation (FMT) from bipolar (BIP) and control (CO) human donors into female C57BL/6 mice. Sucrose preference was measured in metabolic cages starting 7 days post FMT for one week, a social interaction test (SIT) was performed 10 days post FMT, and a forced swim test (FST) and splash test (SIT) at 11 days post FMT. In another cohort, mice were subjected to a light/dark box (LDT) 11 days post FMT and an elevated plus maze (EPM) 13 days post FMT. Mice were sacrificed 14 days post FMT.

Open Field Test

An open field test can be used to assess differences in locomotion between experimental groups of mice. The open field apparatus is a 50 cm × 50 cm × 40 cm opaque, gray plastic box. Mice were placed in one corner of the open field and their locomotion (distance, velocity, speed) was measured for 5 minutes using a video camera and EthoVision software. Additionally, the area of the open field was divided into a 36 cm × 36 cm center zone and the surrounding periphery zone. Entry into either the center zone or periphery zone was defined as the moment when the center point of the mouse (as tracked with EthoVision software) entered the respective zone. The open field box was illuminated by 35 lux. After each session, the open field was cleaned with 70% ethanol.

Barnes Maze

The Barnes maze (BM) test was used to assess spatial learning and spatial memory ability in Pyy-KO mice relative to control mice. The BM test was adapted from the protocol used by Attar et al. 2013[41] with a few changes. The Barnes maze is a circular white polyvinyl chloride platform with a diameter of 91 cm elevated 61.5 cm above the floor. Twenty holes with a diameter of 5 cm were evenly distributed around the

perimeter, 2.5 cm away from the edge. A small, enclosed escape cage (target hole) was attached underneath one of the 20 holes. The BM platform was illuminated by 35 lux. Four visual cues in the form of colored circles and squares on a piece of paper were mounted around the room to allow the test animal to spatially orient on the BM platform.

The animals were put through the BM over the course of three consecutive days. On the first day, the mice were habituated to the BM without the escape hole, and on days 2 and 3 the mice were trained to find and enter the escape hole. This short training phase consisted of two training trials on day 2 and three training trials on day 3. For each training trial, the mice were placed in the center of the maze and allowed to explore for 2 minutes. If the mouse did not find and enter the escape hole, they were guided to it with a glass beaker. If the mouse still did not enter the hole after 1 minute, the mouse was gently nudged into the hole with the beaker. On day 4, a probe session without the escape cage was performed.

The movement of the mice during both training and probe sessions were tracked with a video camera and evaluated with EthoVision software. Spatial learning was quantified by a decrease in the latency to find the target hole (target latency) during the training trials. Spatial memory was determined by the time the mouse spent in the target area (quadrant of BM with target hole in the center) on the probe day. To avoid bias towards any spatial or visual cues, the location of the escape cage was alternated through four different positions on the maze. After each test session the BM and escape cage were cleaned with 70% ethanol.

Novel Object Recognition Test

The novel object recognition test (NORT) was used to assess spatial memory ability in Pyy-KO mice compared to WT mice. The animals were placed in an open field apparatus containing two objects placed across from one other in the center of the field. Mice were given 5 minutes to explore the objects. One hour later, the mice were placed back in the field for 5 more minutes and re-exposed to one familiar object together with a novel object . Each trial was recorded with a video camera. The time of object exploration was assessed with EthoVision software and the performance of each mouse was expressed by the memory index (*MI*) with the formula: $MI = (tn - to)/(tn + to)$. The

time exploring the new object is represented by *tn*, and time exploring the old object is represented by *to*.[42] To avoid any spatial and/or object bias, the position of the objects was alternated between trials, and the “familiar” and “novel” object was changed from mouse to mouse across Pyy-KO and WT groups. After each test session, the objects and open field apparatus were cleaned with 70% ethanol.

Forced Swim Test

The forced swim test (FST) was used to assess depression-like-behavior in mice. The test used was adapted from the protocol used by Painsipp et al. 2011.[43] For the experiment, each test mouse was placed in a glass cylinder (diameter: 16 cm, height: 23 cm) filled with 25°C tap water. The water in the cylinder was 16 cm deep, which ensured that the mice could not touch the bottom of the beaker with their paws or tail. Three categories of behavior–climbing, swimming, and immobility–were scored during each 6 minute test session.[44,45] The time each mouse spent climbing, swimming and floating (immobility) during the FST was expressed as a percentage of the total duration of the test. Mice were considered immobile when they floated passively in the water, moving only in ways that allowed them to keep their heads above the water.[46] Spending more time immobile in the water is considered depression-like-behavior in mice, whereas swimming and climbing signify stress-coping strategies. After the test, the mice were placed under a warming lamp for a few minutes to dry off before being returned to their home cages.

LabMaster Cages

The circadian patterns of locomotion, exploration, sucrose preference, and water and food intake of bipolar and control mice were recorded continuously for one week using the LabMaster system (TSE Systems, Bad Homburg, Germany) 7 days post FMT. The LabMaster system protocol as described by Painsipp et al. 2013 was used.[47] The LabMaster system consisted of six 42 x 26.5 x 15 cm test cages with two external infrared frames and a lid attached to two weight transducers. Cages were filled with regular mouse bedding. These recording units were connected to a computer used to collect and analyze the data with the LabMaster software. The hardware sampling rate

was 100 Hz at the infrared frames and 1 Hz at the drinking and feeding sensors. The sampling interval of the LabMaster software was 1 minute, meaning that recordings were summed up in 1 min intervals. For example, over the course of a 12 hour period, 720 values of each test parameter would be collected.

The two weight transducers were used to measure food and water intake. For feeding, a food bin was filled with standard rodent chow (altromin 1324 FORTI; Altromin, Lage, Germany). Because we also used the Labmaster system to assess sucrose preference, one drinking bottle was filled with tap water and another was filled with a 1% sucrose solution. Both bottles were attached to a transducer on the cage lid. The drinking flasks were equipped with a special nozzle that prevented leakage. However, in case of nozzle malfunction, the Labmaster system was turned on 24 hours before the mice were placed in the test cages in order to check for spontaneous bottle leaking with the LabMaster equipment.

To record locomotion and exploration behavior, the two external infrared frames were placed 4.3 cm apart horizontally, with the lower frame 2.0 cm above the bottom of the cage. The bottom infrared frame recorded horizontal locomotion (ambulatory movements) of the mice, while the top infrared frame recorded vertical movements (rearing, exploration). Mouse activity was measured by counts of interruptions of the light beams of the infrared frames. An ambulatory movement was defined as a successive interruption of any two light beams in one axis, and the total locomotion was determined by adding up the counts in both the x- and y-axes over select time intervals.

Light/Dark Box Test

The light/dark box test is used to assess anxiety-like-behavior in mice. The light-dark box consisted of a cage (37 cm × 21 cm × 20.5 cm, length × width × height) divided into two equally sized sections by a partition containing a door (4.5 cm × 6 cm) (TSE Systems). The light compartment (18.5 × 21 cm) was made of transparent walls and brightly illuminated (350-400 lux), whereas the dark compartment (18.5 × 21 cm) was made of black acrylic walls (20 lux). The mice were put in the light compartment facing the opening to the dark compartment, and locomotion and exploration of the animals were tracked via two external infrared frames, which recorded light beam interruptions

(counts) during a 10-minute test period. These devices were connected to a personal computer which collected and analyzed the data with the LabMaster software (TSE Systems, Bad Homburg, Germany). Transitions between the compartments were counted automatically by the system when the estimated center of gravity of the mouse moved from one compartment to the other and remained in the other compartment for at least 1 second. A shortening of the time spent in the light compartment was taken as an index of anxiety-like-behavior. Since mice are nocturnal and prefer darkness, analysis of the test is based on the premise that more anxious mice will avoid the light compartment and favor the dark compartment more than a healthy mouse.

Social Interaction Test

The social interaction test (SIT) was used to measure social behavior in bipolar and control FMT mice. The test was performed in an open field apparatus containing two wire cages divided by a wooden panel extending halfway down the open field box. The SIT consisted of two phases: a habituation phase and a phase during which the test mouse was exposed to a stranger mouse from a different home cage than the test mouse. During the habituation phase, the test mouse was placed in the open field containing the empty wire cages for 5 minutes. During the stranger mouse phase, the stranger mouse was placed in one of the wire cages, and the test mouse was allowed to explore for 5 minutes. The cage placement (right or left) of the stranger mouse was alternated between sessions. The time the test mouse spent in the vicinity (3 cm radius) of the stranger mouse's cage vs the time it spent in the vicinity of the empty cage was measured with EthoVision software, using the nose point of the test mouse as a reference. Each test mouse was given a one hour interval between the habituation phase and the stranger mouse phase. After each session, the open field apparatus and wire cages were cleaned with 70% ethanol.

Elevated Plus Maze

The elevated plus maze (EPM) is designed to assess anxiety-related-behavior in mice. The apparatus itself is a four-armed, plus-shaped maze made of opaque gray plastic. The maze has two open arms (30 cm × 5 cm) and two closed arms (30 cm × 5 cm with

15.5 cm high, opaque walls). The four arms converged at a center platform (5 cm × 5 cm). The maze was raised 60 cm above the floor and illuminated 35 lux from above. Mice were placed in the center area facing one of the open arms and then were allowed to explore the maze for 5 minutes. After each session, the maze was cleaned with 70% ethanol. The mice were recorded with a video camera and their anxiety, locomotion, and exploration behaviors were analyzed with EthoVision video-tracking software.

Anxiety-related behavior was quantified by the number of entries and time spent in the open arm of the maze. The center point of the mouse was tracked with EthoVision software to determine entry into a specific arm of the maze. This analysis of anxiety-related-behavior is based on the concept that anxious mice tend to avoid the open arms of the maze and spend more time in the closed portion. A less anxious mouse, by contrast, is more likely to explore the entire maze.

Sucrose Preference Test

The sucrose preference test is used to assess depression-like-behavior in mice. The mice in this experiment were given the choice to drink from two bottles, one filled with tap water and one filled with a 1% sucrose and tap water solution. The daily intake of water and sucrose solution was determined by weighing the bottles once a day. In order to control for any behavioral biases, the relative position of the two bottles in the cage lid was switched every day. Mice expressing more depression-like-behavior typically display less sucrose preference than normal mice. The sucrose preference test can also be performed with the LabMaster system.[43]

Splash Test

The splash test is a behavioral test used to assess depression-like-behavior in mice. For the splash test, mice were placed in individual cages and sprayed with a 10% sucrose and tap water solution from a spray bottle. This promotes grooming behavior, as the mice will want to remove the sticky solution from their fur. Grooming behavior was then quantified by latency, duration, and counts of both head grooming and total body grooming. The premise of the test is based on the assumption that mice exhibiting depression-like-behavior will be more neglectful, and groom themselves less than

healthy mice. The mice were recorded with a video camera for 5 minutes after being sprayed, and grooming behavior was tracked with EthoVision software.

Dextran Sulfate Sodium (DSS) Induced Colitis

In order to induce colitis, male mice were treated for 7 days with 2% dextran sulfate sodium (molecular weight 36-50 kDa; MP Biomedicals, Illkirch, France; added to the drinking water). Before DSS treatment, both Pyy-KO and WT mice were subjected to an open field test (OFT), elevated plus maze (EPM), and light/dark box test (LDT). The OFT was performed again on day 1 of DSS treatment, the elevated plus maze (EPM) on day 3, the light/dark box test (LDT) on day 6, and a sucrose preference test on days 6-8. Mice were sacrificed on day 8. During the duration of DSS treatment, a disease activity index (DAI) was used to assess the progression of colitis in the mice. The DAI is a combined score of weight loss compared to initial weight, stool consistency and bleeding. The score was determined as follows: weight loss: 0 (no loss), 1 (1-5%), 2 (5-10%), 3 (10-20%), and 4 (>20%). Stool consistency: 0 (normal), 2 (loose), and 4 (diarrhea). Bleeding: 0 (no traces of blood), 2 (visual pellet bleeding), 4 (bloody perianal region). The minimum DAI score a mouse could receive was 0 and the maximum was 16. In addition, colon length was measured during tissue extraction as an additional indicator of colitis.

Mouse Tissue Extraction

The mice were deeply anesthetized with pentobarbital (150 mg/kg) via intraperitoneal injection (IP). The mice were determined to be completely anesthetized when pinching of the feet and tail did not elicit any reflexes. Blood was then collected via cardiac puncture with a single-use syringe containing 100 µl of 3.8% sodium citrate as anticoagulant. Blood samples were centrifuged at 7000 rpm and 4 °C for 15 min, and then plasma as supernatant was collected and stored at -70 °C until further use. After blood collection, brains were dissected and immediately frozen for 5 seconds in 2-methylbutane on dry ice. Brains were then wrapped in aluminum foil and stored at -70 °C. The colon was also dissected, and divided into distal and proximal sections, and the contents removed. Colon contents and distal and proximal regions were frozen on dry

ice and then stored at -70 °C. This protocol was performed largely as described by Mayerhofer et al. 2016.[48]

RESULTS

Behavioral Effects of Bipolar Fecal Microbiota Transplantation

During the social interaction test (SIT) 10 days post FMT, the control group spent significantly more time in the vicinity of the stranger mouse than that of the empty cage (adjusted p value = 0.0006, two-way ANOVA). By contrast, the bipolar mouse group did not spend significantly more time exploring the stranger mouse than the empty cage ($p > 0.05$, two-way ANOVA) (Fig. 4D). During the LDT 11 days post FMT, bipolar mice spent less average time in the light box than control mice did ($p > 0.05$, unpaired t-test) (Fig. 4C). Thirteen days post FMT, we observed that mice in the bipolar group spent less time on average in the open arm of the EPM than mice in the control group did ($p > 0.05$, unpaired t-test) (Fig. 4A). Bipolar mice also tended to make less visits on average to the open arms of the maze than control mice did ($p > 0.05$, unpaired t-test) (Fig. 4B).

We did not observe many notable differences between bipolar and control mouse behavior during the forced swim test (FST) and the splash test (Fig. 5). No significant differences or notable trends in immobility, swimming, and climbing duration were found during the FST (Fig. 5A). However, bipolar mice displayed a shorter latency to immobility (floating) than control mice did (Fig. 5B). No difference between bipolar and control group grooming duration was found during the splash test (Fig. 5C).

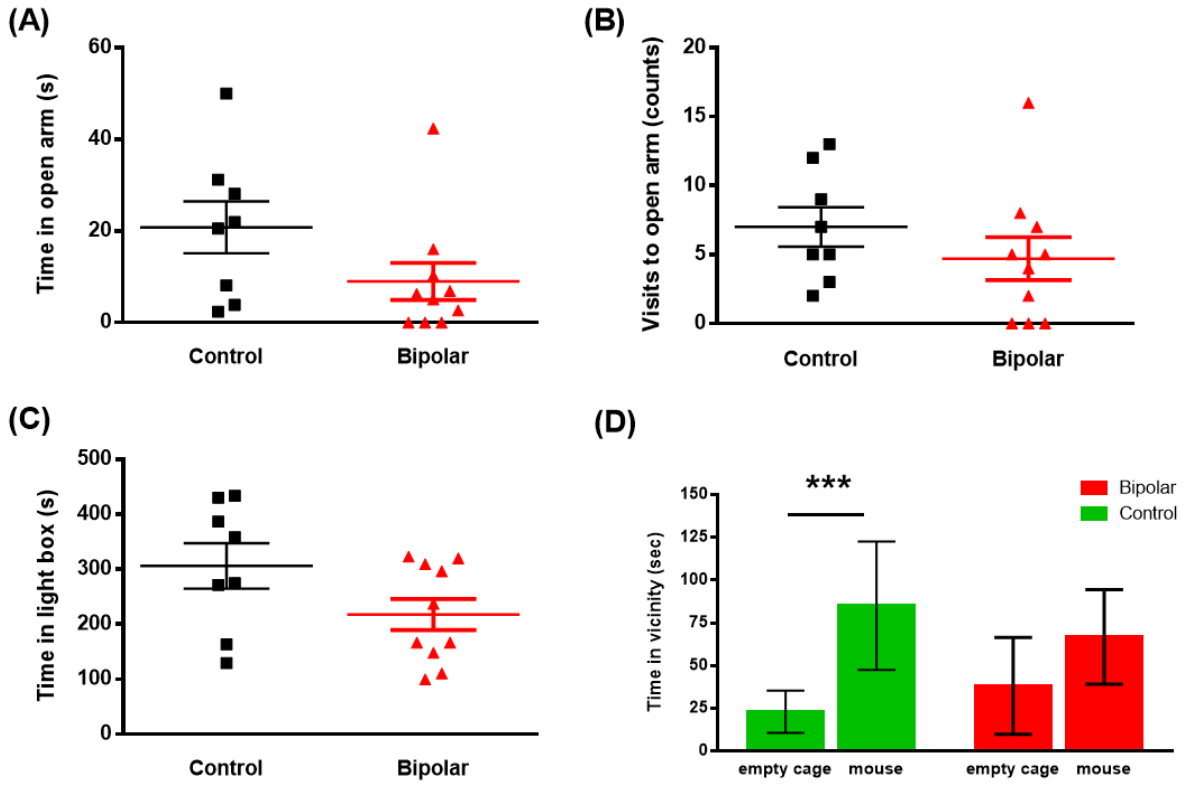

Figure 4. Bipolar mice displayed more anxiety-related-behavior than control mice during the elevated plus maze (EPM) (A-B), light/dark box test (LDT) (C), and social interaction test (SIT) (D). Thirteen days post FMT, bipolar mice spent less average time in the open arm (A), and made fewer visits to the open arm of the EPM than control mice did, although no significant difference was found ($p > 0.05$, unpaired t-test) (B). Eleven days post FMT, bipolar mice spent less time on average in the light compartment of the LDT, although the difference was not found to be significant ($p > 0.05$, unpaired t-test) (C). During the SIT performed 10 days post FMT, control mice spent significantly more time in the vicinity of test mouse than the empty cage (adjusted p value = 0.0006, two-way ANOVA), whereas the bipolar group did not ($p > 0.05$, two-way ANOVA) (D).

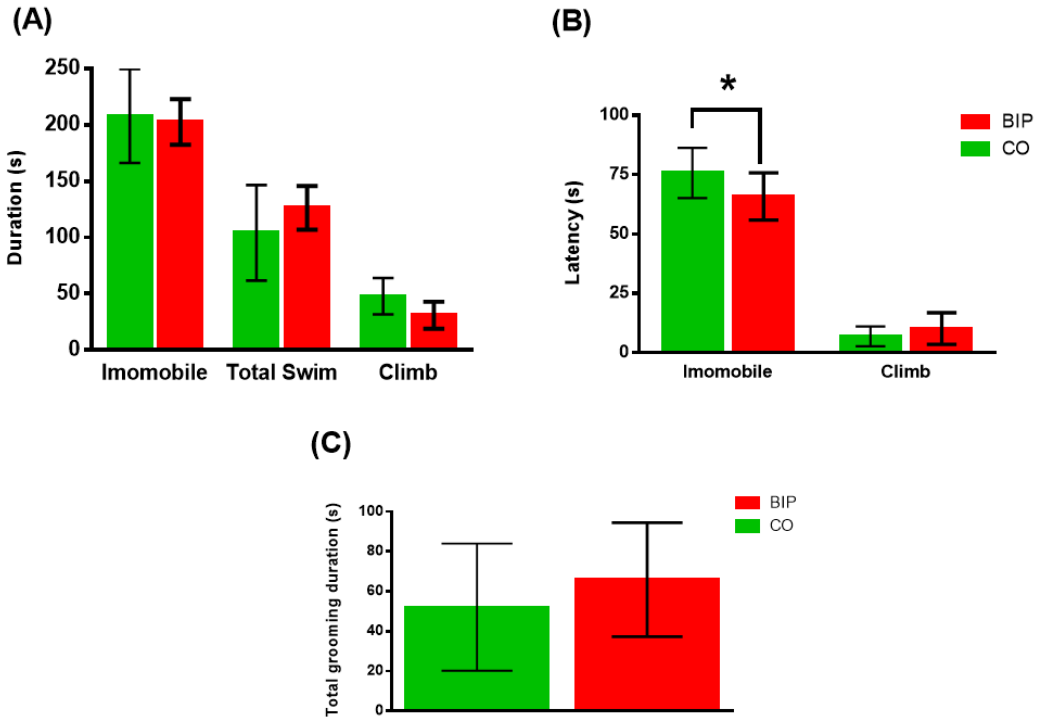

Figure 5. Less behavioral differences were observed between bipolar and control mice during the forced swim test (A-B) and the splash test (C) performed 11 days post FMT. There was no significant difference ($p > 0.05$, two-way ANOVA) immobility, swimming, and climbing duration (A) between bipolar and control FMT mice. There was a significant difference ($p < 0.05$, two-way ANOVA) in latency to immobility, wherein bipolar mice were quicker to commence passive floater than control mice. There was no significant difference ($p > 0.05$, unpaired t-test) in grooming duration between bipolar and control groups (C).

Spatial Learning and Memory Abilities of Pyy-KO Mice

My data indicated that Pyy-KO mice may have reduced spatial memory and learning abilities relative to WT mice. Although no significant differences were found between Pyy-KO and WT groups during the training ($p > 0.05$, two-way ANOVA) (Fig. 6A) and probe trials ($p > 0.05$, unpaired t-test) (Fig. 6B) of the Barnes maze (BM), there was an observable trend that the Pyy-KO group on average exhibited increased latency to find the target hole relative to WT mice during the training trials, especially on days 3 and 4. The Pyy-KO mice also spent less time on average in the target quadrant during the probe trial than the WT mice did. The novel object recognition indicated the same trend, with Pyy-KO mice having a lower object recognition memory index ($MI = (tn - to)/(tn +$

to)) than control mice (Fig. 6C). However, no significant difference in memory index was found between WT and Pyy-KO groups ($p > 0.05$, unpaired t-test).

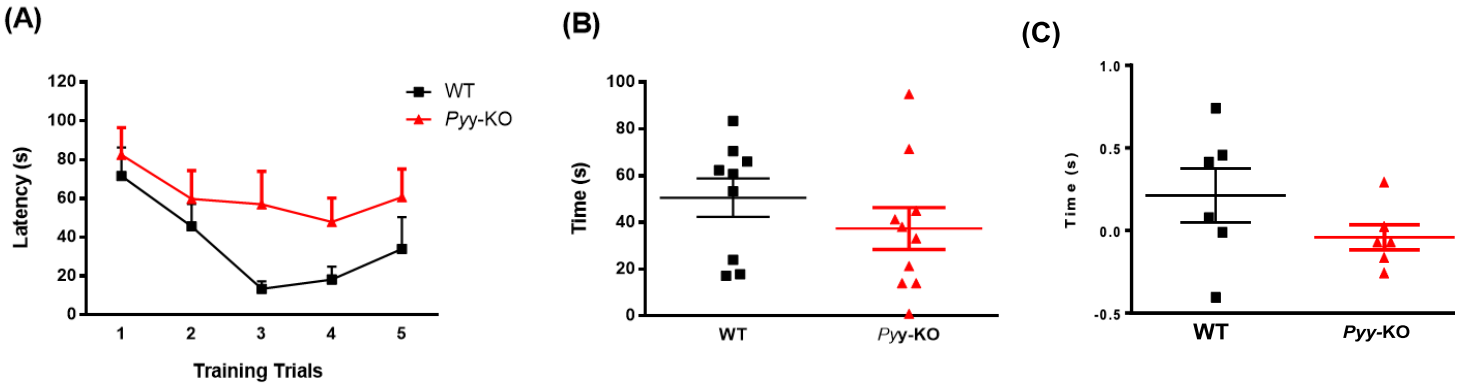

Figure 6. The effect of a lack of PYY signaling on spatial learning and memory abilities as determined by the Barnes maze (BM) test (A-B) and novel object recognition test (NORT) (C). Latency to target hole during training (A) and duration in target quadrant during probe trial (B) of BM. NORT memory index = (novel - familiar object)/ total time exploration during test) (C). No significant differences were found between Pyy-KO and WT groups during training ($p > 0.05$, two-way ANOVA) and probe trials ($p > 0.05$, unpaired t-test) of the BM; however, trends indicate that Pyy-KO mice may have reduced spatial and learning memory relative to WT mice. No significant difference in memory index was found between WT and Pyy-KO groups during NORT ($p > 0.05$, unpaired t-test), but it was observed that Pyy-KO mice had reduced recognition of the novel object (lower memory index) compared to WT mice (C).

DSS treatment caused more severe colitis in Pyy-KO mice

Disease activity index (DAI) scoring over the course of the 7 day dextran sulfate sodium (DSS) treatment showed that Pyy-KO mice experienced worse colitis symptoms than WT mice (Fig. 7J). Neither group showed colitis symptoms until day 3 of treatment. On day 3-5, the Pyy-KO mice had higher DAI scores than WT mice, with Pyy-KO mice losing more weight, experiencing more diarrhea and softer stool, and displaying more visual gross anal bleeding than WT mice.

The Effects of DSS-Induced Colitis on Emotional Behavior in Pyy-KO Mice

On day 1 of dextran sulfate sodium (DSS) treatment, WT and Pyy-KO mice were subjected to an open field test (OFT). Compared to pre DSS OFT results, WT mice

traveled a significantly greater distance ($p < 0.05$, two-way ANOVA) (Fig. 7A), spent more time in the center zone (Fig. 7B), and made more visits to the center zone of the OFT during DSS treatment (Fig. 7C). By contrast, the distance traveled, time spent in center zone, and number of center zone visits post DSS treatment was not significantly different in the Pyy-KO group ($p > 0.05$, two-way ANOVA).

On day 3 of DSS treatment, Pyy-KO mice spent about 50% less time in the open arm of the elevated plus maze (EPM) than they did before DSS treatment ($p > 0.05$, two-way ANOVA) (Fig. 7D). Pyy-KO mice also displayed a significant reduction in open arm visits during DSS treatment ($p < 0.05$, two-way ANOVA). The Pyy-KO group made significantly more visits to the open arm than WT pre DSS treatment ($p < 0.05$, two-way ANOVA) (Fig. 7E). WT mice traveled significantly less distance in the EPM on day 3 of DSS treatment than they did before treatment ($p < 0.05$, two-way ANOVA). The EPM distance traveled by the Pyy-KO group during DSS was not significantly different from the distance traveled pre-treatment ($p > 0.05$, two-way ANOVA) (Fig. 7F).

Time spent in the light compartment of the light/dark box did not change significantly on day 6 of DSS treatment for both WT and Pyy-KO groups ($p > 0.05$, two-way ANOVA) (Fig. 7G). Pre DSS treatment, Pyy-KO mice displayed significantly more rearing behavior than WT mice ($p < 0.05$, two-way ANOVA). Rearing counts were not significantly affected by DSS treatment in either group ($p > 0.05$, two-way ANOVA) (Fig. 7H). During DSS, Pyy-KO mice traveled significantly less distance during the light/dark box test than they did before treatment ($p < 0.05$, two-way ANOVA) (Fig. 7I). The Pyy-KO group had a significantly higher disease activity index (DAI) score of colitis on day 5 of DSS treatment than the WT group ($p < 0.05$, unpaired t-test) (Fig. 7J).

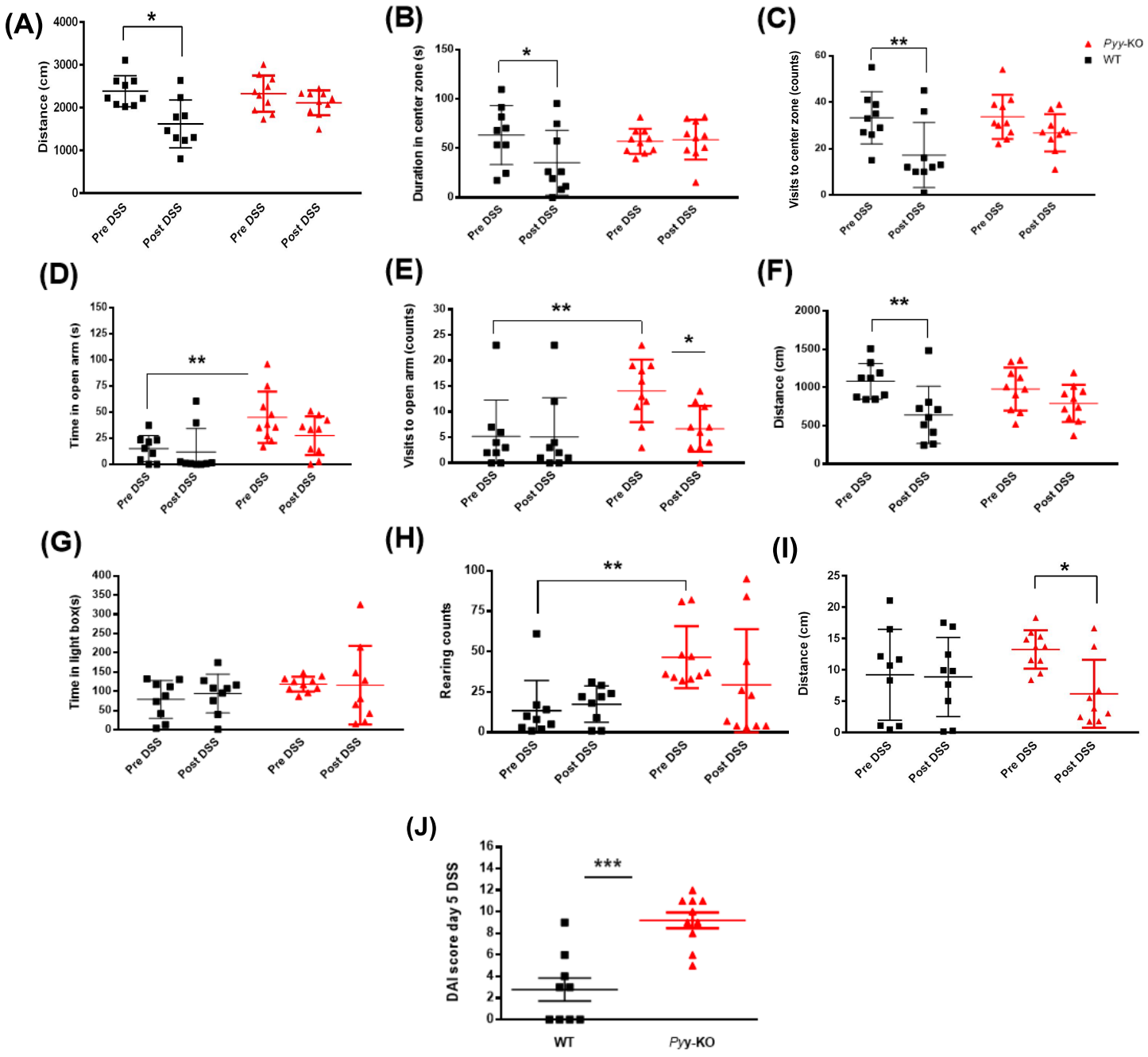

Figure 7. On day 1 of DSS treatment, WT mice displayed a significant decrease in distance moved ($p < 0.05$, two-way ANOVA) (A), spent a longer time in center zone (B), and increased their number of visits to the center zone of the OFT relative to pre DSS treatment (C), whereas the behavior of Pyy-KO mice did not change significantly after 1 day of DSS treatment ($p > 0.05$, two-way ANOVA). On day 3 of DSS, Pyy-KO mice underwent a greater decrease in time spent in the open arm of the EPM than WT mice (D). Pyy-KO mice also made significantly less open arm visits than they did before DSS treatment ($p < 0.05$, two-way ANOVA). Pre DSS treatment, Pyy-KO mice made more visits to the open arm than WT mice ($p <$

0.05, two-way ANOVA) (E). WT mice exhibited a significant decrease in distance traveled during the EPM post DSS ($p < 0.05$, two-way ANOVA), but Pyy-KO mice did not ($p > 0.05$, two-way ANOVA). Neither WT nor Pyy-KO groups spent significantly more time in the light compartment of the LDT post DSS ($p < 0.05$, two-way ANOVA) (G). Pre DSS treatment, Pyy-KO mice displayed more rearing behavior than WT mice ($p < 0.05$, two-way ANOVA). Rearing counts were not significantly affected by DSS treatment in either group ($p > 0.05$, two-way ANOVA) (H). Post DSS, Pyy-KO mice traveled less distance during the light/dark box test ($p < 0.05$, two-way ANOVA) (I). The Pyy-KO group had a higher disease activity index (DAI) score of colitis on day 5 of DSS treatment than the WT group ($p < 0.05$, unpaired t-test) (J).

DISCUSSION

For my Fulbright research project, I took a “bottom-up” approach to studying the gut-brain axis by focusing on how alterations in GI tract affect the brain, particularly in regards to emotional behavior. For one part of this project, I researched how fecal microbiota transplants (FMT) from a human patient with bipolar disorder affected behavior in mice relative to mice that had received FMT from a healthy control. This research has the potential to help us understand the role of the gut-brain axis in mental health conditions like bipolar disorder, and could play an important role in the development of new treatments. I also looked into how a lack of peptide YY (PYY) signaling in mice affects cognitive processes like spatial learning and memory, as well as emotional response to immune stress. Peptide YY is regulated my gut microbiota, so determining the different ways that PYY affects behavior will help us better understand the complexities of the gut-brain axis.

Behavioral Differences Between Bipolar and Control FMT Mice Observed

The results of the elevated plus maze (EPM) and light//dark box test (LDT) indicated that bipolar FMT mice exhibit more anxiety-related-behaviors than control FMT mice. For the light/dark box test 11 days post FMT, bipolar mice spent less time on average in the light compartment than control mice did (Fig. 4C). During the EPM at 13 days post FMT, bipolar mice were observed to spend less time in the open arm of the EPM (Fig. 4A). Bipolar mice also made fewer visits to the open arm than control mice did (Fig. 4B). These findings indicate that bipolar mice exhibit more anxiety-related-behavior than

control mice because healthy mice are more emboldened to explore the brightly lit areas of the open arms of the EPM and the light compartment of the light/dark box than an anxious mouse.

The results of the social interaction test (SIT) at 10 days post FMT revealed differences in social behavior between bipolar and control mice (Fig. 4D). The control mice spent significantly more time interacting with the stranger mouse ($p = 0.0006$, two-way ANOVA) than with the empty cage. By contrast, the bipolar group did not spend significantly more time exploring the stranger mouse than the empty cage ($p > 0.05$, two-way ANOVA). This disinterest in interacting with the stranger mouse suggests that the bipolar group was exhibiting more depression-like-behavior than the control group.

There are some inconsistencies in the behavior of FMT mice that are worth noting. The results of a previous LDT and EPM test performed with male mice found that while bipolar mice spent less time on average than control mice in the open arm of the EPM 11 days post FMT, they spent more time in the light compartment during an LDT performed 13 days post FMT. For my experiment, we performed the LDT and EPM in a different order, with the LDT at 11 days post FMT and the EPM at 13 days post EPM. However, bipolar mice displayed more anxiety-like-behavior for both tests. It is possible that these behavioral inconsistencies are a factor of bipolar FMT in mice. However, future trials will need to be done in order to determine whether these discrepancies are part of the manifestation of bipolar disorder in mice post FMT or are due to some other factor. Regardless, the results of my study show that FMT from a human donor with bipolar disorder is correlated with behavioral changes in mice.

Even though I have completed my role in this project, additional research and analysis will be done before publishing the results. The bacterial taxa present in bipolar and control mice will be analyzed and compared. Differences in neural activity will also be assessed in the brains of bipolar and control mice. Data on the metabolites present in both groups have also been collected and are currently being analyzed. Interestingly, it was found that colonic expression of PYY was decreased in bipolar mice relative to control mice, demonstrating the connectedness of gut-brain axis research.

This study is just one step in understanding the complexities of the microbiome and gut-brain communication. However, I believe that this translational work will help lay the foundation for understanding the role of the gut-brain axis in mental health conditions like depression and bipolar disorder.

Pyy-KO Mice May Have Reduced Spatial Memory and Learning Capability

I also studied the involvement of the gut-microbiota regulated peptide, peptide YY (PYY), in brain function, particular in regards to memory. To do this, I conducted behavioral tests such as the Barnes maze (BM) and a novel object recognition test (NORT) that assessed the spatial learning and memory abilities of Pyy-KO mice relative to wildtype mice. The results of the BM and the NORT indicated that Pyy-KO mice have worse spatial learning and memory abilities than WT mice (Fig. 6). Although the differences were not statistically significant ($p > 0.05$, two-way ANOVA), it was observed that over the course of BM training, Pyy-KO mice were slower to find the target hole relative to WT mice, especially on days 3 and 4 (Fig. 6A). These findings suggest that the spatial learning of Pyy-KO is worse than that of WT. Additionally, Pyy-KO mice were slower to find the target hole during the probe trial of the BM (Fig.6B) and worse at recognizing the novel object during the NORT (Fig. 6A), showing that spatial memory is also weaker in Pyy-KO mice compared to WT.

Results of Behavioral Tests during DSS are Conflicting

After inducing colitis in Pyy-KO and WT mice using a dextran sulfate sodium (DSS) treatment, I performed an open field test, elevated plus maze, and light/dark box test in order to assess the emotional behavior of both groups during immune stress. The DSS treatment caused more severe colitis in Pyy-KO mice than it did in WT mice (Fig. 7J). On days 3-5 of DSS, Pyy-KO mice had greater DAI scores. The health of Pyy-KO mice was also visibly worse, with Pyy-KO mice exhibiting more weight loss, softer stool, and more perianal bleeding.

The behavioral responses of Pyy-KO and WT mice during DSS treatment were less clear. We expected that a lack of PYY signaling would lead to more anxiety-like-behavior during immune stress, but this was not always the case. On day 1

of DSS treatment, WT mice exhibited a greater decrease in duration spent in the center area and visits to the center area than Pyy-KO mice (Fig. 7A-C). However, since neither group exhibited symptoms of colitis on day 1 of treatment, it is unlikely that these results indicate a difference in behavioral response to immune stress. The OFT results do potentially indicate that Pyy-KO and WT mice habituated differently to the open field, with WT mice exhibiting less exploratory behavior during the second session than Pyy-KO mice. This could potentially be explained by my previous finding that Pyy-KO mice have reduced spatial learning and memory capabilities than WT mice. As a result, WT mice that remembered the OFT course might be less inclined to explore than Pyy-KO mice with a worse memory of it.

Before DSS treatment, Pyy-KO mice spent more time in and made more visits to the open arm of the EPM than WT mice (Fig. 7D). These results are somewhat surprising given that other studies have found that Pyy-KO mice display more anxiety-like-behavior than WT mice.[38] However, we used a different strain of Pyy-KO mice in this experiment. During experimentation, I also noticed that these Pyy-KO mice were more restless than WT mice, which potentially resulted in increased exploratory behavior. It is unclear why this strain of Pyy-KO mice behave differently, and will be a point of future investigation.

After DSS treatment, Pyy-KO mice spent approximately 50% less time in the open arm of the elevated plus maze (EPM) than they did before DSS treatment ($p > 0.05$, two-way ANOVA) (Fig. 7D). Pyy-KO mice also significantly reduced their visits to the open arm during DSS treatment ($p < 0.05$, two-way ANOVA). By contrast, open arm duration and visits did not change in the WT group (Fig. 7E). These results indicate that on day 3 of DSS, Pyy-KO mice may have been exhibiting more anxiety-behavior than WT mice. However, these results are not consistent with the finding that WT mice traveled a significantly shorter distance in the EPM during DSS treatment, whereas distance traveled was not significantly changed in the Pyy-KO group during treatment (Fig. 7F). This behavior could also be explained if Pyy-KO mice have worse spatial learning and memory than WT mice; the WT may have remembered the EPM course better than the Pyy-KO mice, and therefore explored less.

For the LDT on day 6, DSS treatment did not seem to affect light compartment duration and visits in either group(Fig. 7G). Pyy-mice exhibited a greater decrease in rearing counts during DSS treatment than WT mice did (Fig. 7H). Rearing is an exploratory behavior, so a lack of rearing indicates greater anxiety in mice. Pyy-KO mice also traveled a significantly shorter distance during DSS treatment than they did before, whereas distance traveled by WT mice was unchanged during DSS treatment (Fig. 7I). This was likely a result of Pyy-KO mice having poorer health and more severe colitis than WT at this point in the experiment.

Due to the observation that differences in learning and memory between Pyy-KO and WT groups may have played a role in the results of the behavioral tests, I would recommend repeating this study without subjecting the mice to the OFT, EPM, and LDT before DSS treatment. I would also look into why different stains of Pyy-KO mice might behave differently.

LIST OF ABBREVIATIONS

BBB - blood brain barrier
BD - bipolar disorder
BIP - bipolar
BM - Barnes maze
CNS - central nervous system
CO - control
DSS - dextran sulphate sodium
EPM - elevated plus maze
FMT - fecal microbiota transplant
FST - forced swim test
GBA - gut-brain axis
GI - gastrointestinal
HAM-D - Hamilton Depression Scale for Depression
Hz - Herz
LDT - light/dark box test
NORT - novel object recognition test

NPY - neuropeptide Y

PYY - peptide Y

KO - knockout

SIT - social interaction test

ST - splash test

WT - wildtype

YMRS - Young Mania Rating Scale

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